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03021581.8

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Application no.:   03021581.8
Demande no:

Anmeldetag:
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Anmelder/Applicant(s)/Demandeur(s):

Krammer, Peter
Werderstrasse 11
69120 Heidelberg
ALLEMAGNE

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Antibodies against Annexins and the use thereof for therapy and diagnosis

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DIPL.-FORSTW. DR. **J. LACHNIT**

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Anmelder:

Peter Krammer
Werderstraße 11

D-69120 Heidelberg

Antibodies against Annexins and the use thereof for therapy and diagnosis

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Antibodies against Annexins and the use thereof for therapy and diagnosis

Description

Apoptosis constitutes the most common form of cell death throughout the lifespan of an organism. During development and adulthood the removal of excess cells by apoptosis ensures tissue homeostasis (Wyllie, Kerr, and
5   Currie 251-306;Jacobson, Weil, and Raff 347-54). To preclude the release of noxious cellular contents, dying cells are rapidly cleared by neighbouring phagocytes, e.g. macrophages or dendritic cells. This efficient and safe uptake is mediated via specific signals or ligands, which must provide for engulfment as well as suppression of immune responses against self
10   antigens (Savill et al. 965-75;Steinman et al. 15-25). In order to discover new molecules involved in the signaling of apoptotic cells towards phagocytes, we immunized mice with apoptotic Jurkat cells and generated monoclonal antibodies. One antibody recognized a protein on the surface of early apoptotic cells, which was identified as Annexin I upon sequencing. Blocking
15   experiments with the antibody in cocultures of dendritic and apoptotic cells revealed a role for Annexin I in the suppression of pro inflammatory dendritic cell cytokine release. Thus, our results indicate that Annexin I acts as an anti-inflammatory signal, which suppresses the activation of immune responses towards self antigens.

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Upon apoptotic stimuli such as cytotoxic agents e.g. staurosporine,   -
irradiation or the engagement of the CD95 (APO-1/Fas) receptor, cell death proceeds via activation of caspases, finally leading to the formation of membrane enclosed apoptotic bodies. The efficient removal of these
25   apoptotic remnants by phagocytes prevents the release of intracellular contents, which might otherwise serve as danger signals, resulting in DC activation and initiation of immune responses (Refs Polly). In addition, apoptotic cells have been reported to mediate anti-inflammatory signals to phagocytosing cells, thereby actively down regulating the immune response

(Refs Savill&Fadok, Steinman). Thus, in contrast to necrotic cell death, apoptosis causes little, if any, immune response.

We immunized mice with apoptotic Jurkat T cells (J16) and generated hybridomas to detect new anti-inflammatory signals on the surface of apoptotic cells. One of these antibodies, termed DAC5 for "Detector of Apoptotic Cells", recognized a protein on the surface of early apoptotic but not live Jurkat cells (Fig 1a). Binding kinetics of DAC5 resembled the kinetics of the widely used early apoptosis marker Annexin V. Notably, DAC5 binding occurred before membrane integrity of the cells was lost, as measured by propidium iodide (PI) uptake. This indicates that the antigen was localized on the cell surface. Binding was not restricted to the apoptotic stimulus nor to celltype, as binding occurred also after treatment with leucine zipper CD95 (LZ-CD95), which triggers the CD95 (APO-1/Fas) pathway, as well as upon γ-irradiation (Fig 1b). Cells binding DAC5 upon apoptosis included primary human T cells, HeLa cells and the melanoma cell line A375 (data not shown).

To identify the antigen recognized by DAC5 we performed an immunoprecipitation with purified DAC5 and lysates of J16 cells, that had been treated with 1 μM staurosporine for 4 h or left untreated. In each case, the antibody specifically precipitated a protein of about 37 kDa, as detected on a silver stained SDS PAGE (Fig. 2a). Both bands were cut from the gel, subjected to analysis via mass spectroscopy and identified as Annexin I. Annexin I belongs to a large and well conserved family of proteins that bind to negatively charged phospholipids such as phosphatidyl serine and phosphatidic acid in a calcium dependent manner (Ref). Although studied in detail, the physiological function of the annexin family remains largely elusive (Gerke and Moss. 331-71). Nevertheless, Annexin I has been implicated in the suppression of inflammation before. While originally described as an intracellular mediator of glucocorticoid actions (Solito et al. 1675-83), an extracellular form of Annexin I has been shown to regulate neutrophil extravasation through interaction with the formyl peptide receptor (Walther, Riehemann, and Gerke 831-40). In addition, mice deficient for Annexin I show increased and aberrant inflammation (Hannon et al. 253-55), adding

further evidence to a role for Annexin I in the control of inflammation.

Because Annexin I resides in the cytoplasm of intact cells, we presumed a translocation to the outside of the cell membrane during apoptosis, where it binds to the exposed phosphatidyl serine (Ref). To confirm that Annexin I was indeed present at the surface of apoptotic cells, we performed a washing experiment. The T cell line CEM was incubated with 1 µM staurosporine (sts) for different time points. Subsequently the cells were centrifuged to obtain the supernatant, while the pellets were washed in EDTA-containing PBS to release the calcium-dependent membrane bound Annexin I. Finally, the cells were lysed and Annexin I within each fraction was detected on Westernblot. Upon prolonged incubation times, we detected increasing amounts of Annexin I, that were released from the cell surface (Fig. 3). Accordingly, the intracellular amount of Annexin I was decreased, while the supernatants did not contain any significant amount of Annexin I. This demonstrates that Annexin I was not secreted, but upon translocation immediately bound to the cell membrane.

DCs are the most potent antigen presenting cells and their activation and maturation are critical for the outcome of an immune response (Ref). Therefore, we investigated how Annexin I on the surface of apoptotic cells influenced immature dendritic cells. As has been shown before (Stuart et al. 1627-35), a fraction of the DCs cells readily engulfed fluorescence labeled apoptotic cells, leading to an increase in FL-3 (Fig 4a). Furthermore, we observed less uptake of apoptotic cells that had been coated with DAC5 F (ab')₂ fragments. This confirms a recent report by Arur et al., describing Annexin I as an "eat me" signal for phagocytes (Arur et al. 587-98). Because dendritic cells influence immune responses via the release of cytokines as well as upregulation of cell surface markers, we next measured the levels of the pro inflammatory cytokines TNFα and IL-12 after coincubation of DCs with apoptotic cells. As a control we also incubated necrotic cells with DCs. While necrotic cells directly induced the release of TNFα and IL-12, this was not the case when DCs engulfed apoptotic cells (Fig. 4b). Upon blockade of Annexin I through DAC 5 F(ab)₂-fragments, however, we detected a

remarkable increase in TNFα as well as IL-12 levels (Fig 4c). This effect was not due to LPS contamination, as incubation of DCs with 10 µg/ml DAC5 F (ab')₂ fragment alone did not cause any TNFα or IL-12 release. Although background levels of both cytokines changed dependent on the donor, blocking of Annexin I reproducibly induced an x-fold increase. Remarkably, DC activation markers were not downregulated but rather upregulated in cells that had engulfed apoptotic cells (Fig 4c). Thus, the "silencing" of the immune system by apoptotic cells may depend on suppression of proinflammatory cytokines as well as on additional ligands, upregulated on DCs after apoptotic cell ingestion. One canditate ligand, involved in the maintenance of T cell anergy, has recently been described (Ref Stöckl).

Clearly, the involvement of Annexin I in the removal of apoptotic cells and immune regulation warrants further investigation.

In this respect, Annexin I might well serve a dual function, ensuring at the same time rapid as well as safe removal of apoptotic cells. Because expression of Annexin I is tissue specific as well as developmentally regulated (Dreier et al. 137-48;Della et al. 206-17), cell specific levels of the protein might be adjusted according to a changing turnover rate. Alternatively, other members of the annexin family might fulfill similar anti-inflammatory functions as Annexin I in a tissue specific manner. Unique functions of Annexin I like receptor binding have been attributed to the N-terminus

Our results indicate a role for Annexin I in the induction of a "tolerogenic" DC phenotype towards self antigens. Exploiting this function, the administration of Annexin I or the blocking via antibodies might be beneficial for patients during transplantation and cancer, respectively.

From above-described studies and the studies which are presented in the following examples, several applications and uses can be deduced for annexins in general, especially Annexin I and further also Annexin V, as well as anti-Annexin antibodies, especially anti-Annexin I antibodies or anti-Annexin V antibodies.

Accordingly, anti-Annexin antibodies are a first subject of the present invention. Especially preferred antibodies of the present invention

include anti-Annexin I and anti-Annexin V antibodies. Such antibodies can be used for example and without being limited thereto, for the detection of apoptosis in vivo. This might be very important in cancer therapy, for example for monitoring the progress of an anti-tumoral therapy and further detection whether a tumor is amendable to such therapy. Also the antbodies can be used for a diagnosis of diseases with committant increased apoptosis rate of cells, as for example diabetes and auto-immune diseases. Especially preferred is the use of the anti-Annexin I antibody for the detection of apoptosis in vivo and for the monitoring of therapy. The antibody is according to a preferred embodiment of this invention labeled in such a way that a diagnosis in vivo can be performed, labels which are suitable therefor are known to the man in the art as well as methods for detecting this label.

Other diseases which also show increased apoptosis are for example cardiovascular diseases. Such diseases can be diagnosed as well as treated by an antibody according to the present invention. Especially preferred is the use of an antibody against Annexin I for such diseases.

In the field of cancer therapy, the use of the present antibody can also support a common therapy. Common tumor therapies often are able to destroy a lot of tumor cells, but it is known that for some reasons there are cells that are resistant to tumor therapy and cannot be destroyed. Such cells might be the cause for further tumor growth after the first therapy has been terminated. The use of the present antibody for tumor therapy can avoid such later growth of tumors from resistant tumor cells. The antibody according to the invention for such uses is coupled to an effector molecule, which is able to destroy tumor cells like for example radioactivity or toxic substances. Such substances can be very specifically directed to the tumor locus by using the antibody according to the present invention, and such antibodies bind to tumor cells which due to apoptosis show Annexin I on their surface. The effctor substance which is coupled to the antibody can destroy not only the cell to which the antibody is coupled via Annexin I, but also destroys cells in the environment of such apoptotic cells, which themselves are not yet apoptotic or do not surrender to apoptosis for whatever reason. Therefore,

the use of te present antibody for treating tumors, either alone or in combination with common tumor therapies, leads to the result that the tumor is completely destroyed and no resistant cancer cells can survive such treatment. In such treatment the apoptotic cells showing Annexin I on their surface are bound specifically and destroyed by the effector agent, the cells surrounding such apoptotic cells, however, are also destroyed due to the effect of the effector molecule. The effector molecules can be selected according to their effeciency and their range of effectiveness.

Use of the antibody according to the present invention in therapy and especially in tumor therapy as well as in support of commonly used tumor therapies is also mediated by the following mechanism:

cells showing Annexin I on their surface lead to blockation of inflammatory signals within the immune system. Therefore, such cells are not destroyed efficiently by the immune apparatus. When antibodies bind to the Annexin I molecules, they block the anti-inflammatory signals that are presented to the immune effector cell. Therefore, an inflammation can take place which itself supports the immune response in for example cancer therapy as well as support of an immune response against self-antigens.

For such purpose the antibodies need not necessarily be labeled, whereas for other therapeutic or diagnostic methods it is preferred to use a labeled antibody. This label can be a molecule which allows for detection of the bound antibodies in vitro or in vivo or a substance that is able to destroy cells, like for example radioactivity or a toxic substance. As mentioned above, such substances which might be coupled to the antibodies according to the present invention are known to the man in the art.

Another subject of the present invention is the use of Annexin I for certain indications. From the studies connected to the present invention, it could be gathered that Annexin I, when presented on the cell surface, gives an anti-inflammation signal. Such anti-iinflammation signals also occur when foreign tissues are transplanted into a patient. The use of Annexin I can avoid such problem when administered to a patient or to tissue to be transplanted. Presentation of Annexin I on such tissues leads to the result that the immune

response does not recognize such foreign tissue, but rather takes it as non-foreign tissues and therefore no inflammatory response is initiated. Administration of Annexin I or fragments thereof therefore leads to a marking of such transplantation tissue that avoids an inflammatory effect. Immune
5 response against such transplanted tissue can thus be suppressed.

This effect obviously is true also for other annexins, wherefor use and/or application of other annexins to transplantation patients or tissue to be transplanted is encompassed by the present invention, especially Annexin V, as another means according to the present invention to avoid
10 inflammatory immune reaction against the transplanted tissues.

A further possibility of using the results of the present invention is that Annexin I and other annexins are related to specific receptors, which again can be stimulated or blocked by either binding of one of the annexins or an antibody against this receptor. Such binding of receptors by an annexin
15 or an antibody against the annexin-receptor can also influence the immune response and lead to the same possibilities for therapy as described above for the annexin antibodies or the use of annexin in transplantation.

The following example and figures are meant to further illucidate the present invention.
20

Example

Immunization and fusion – C57BL/6 mice were kept under certified pathogen free conditions and immunized nine times i.p. with $2x\ 10^7$ apoptotic J16 Jurkat T cells. Apoptosis was induced by incubation with 1 µM
25 staurosporine for 0.5 h, and the cells were thoroughly washed in sterile PBS before injection. During the first 3 immunizations, the apoptotic J16 cells were coated with 1,2 µg/ml recombinant hu Annexin V (sigma) to promote generation of high affinity antibodies (Stach et al. 911-15). Hybridomas were generated from the spleens by Polyethylene glycol-induced fusion with the
30 mouse plasmacytoma line Ag8.

Cell lines and hybridomas - The human T-ALL cell line CEM and the human Jurkat T cell line J16 were cultured in RPMI 1640 (Gibco)

supplemented with 10% fetal calf serum (Gibco) and 10 mM HEPES (Sigma). Supernatant of the hybridoma DAC 5 was affinity purified using a Protein A–Sepharose column (Sigma), followed by dialysis against PBS. Fab and F(ab')$_2$ fragments of DAC5 were generated using Immunopure kits (Pierce) according to manufacturers instructions.

Preparation of primary human DCs and primary human T cells - Human immature DCs were obtained as described previously (Ref). Briefly, monocytes were prepared by adherence from 300 – 500 ml whole blood of healthy donors. Cells were then washed with PBS and plated at 1×10^6 cells/ml in X-Vivo medium (BioWhittaker), containing 1000 U/ml hu GM-CSF (Leucomax) and 800 U/ml hu IL-4 (R&D). Fresh cytokines were supplemented again on day 4. Immature DCs were used on day 7, and >98% were positive for the DC marker CD11c. Human peripheral T cells were prepared as described previously (Klas et al. 625-30). After activation with 1µg/ml PHA, T cells were incubated for another 5 days in RPMI, supplemented with 25 units/ml IL-2 (Ref).

Immunoprecipitation and silver staining – For immunoprecipitation, 1×10^7 J16 cells were lysed in Triton X-100 Lysisbuffer (20 mM Tris/HCl, pH 7.4, 1 % Triton X-100, 10 % glycerol, 150 mM NaCl, 1 mM PMSF and 1 µg/ml of Leupeptin, Antipain, Chymostatin and Pepstatin A) for 15 min on ice and centrifuged (15 min, 14000xg, 4°C). Supernatants were then subjected to immunoprecipitation with 10 µg DAC5 or isotype control antibody and Protein A Sepharose at 4°C overnight. Precipitates were prepared in SDS sample buffer, resolved on a 12% SDS PAGE and stained with the Silverquest silver staining kit (Invitrogen), according to manufacturers instructions.

Mass spectroscopy – kommt montag von martina

Surface staining - 5×10^5 cells were incubated with 100 µl of hybridoma culture supernatant or with 10 µg/ml purified DAC5 in PBS/10% FCS for 30 min at 4°C, washed with 500 µl PBS/10% FCS, and incubated another 30 min with 5 µg/ml FITC-labeled goat anti mouse IgG antibodies (Dianova)

and 2 µg/ml PI (Molecular Probes) in PBS/10% FCS. The addition of 10% FCS provided for calcium, necessary for Annexin I to bind to the membrane of apoptotic cells. After further washings, the cells were analyzed on a FACScan cytometer (Becton Dickinson). Annexin V-FITC was purchased from Molecular Probes and staining was done according to manufactures instructions in Annexin Binding buffer (10 mM HEPES, 140 mM NaCl, 2,5 mM $CaCl_2$, pH 7,4).

EDTA-washes and Westernblot analysis – $5x10^6$ CEM cells were incubated in 500 µl medium with 1 µM staurosporine for different time periods or left untreated. Subsequently, the cells were centrifuged, the supernatant was collected and pellets were washed with 500µl PBS/10mM, EDTA. After centrifugation the washing solution was collected as well, and the pellets were lysed in 500 µl Triton X-100 Lysis buffer. A 200 µl aliquod of each fraction was separated on a 12 % SDS-PAGE, blotted onto nitrocellulose membrane (Amersham) and detected with monoclonal antibodies against Annexin I (Transduction).

Engulfment of apoptotic cells by DCs – J16 cells were labeled with fluorescent DiD (Molecular Probes) according to manufacturers instructions. Immature d6 DCs were plated at a density of $2x10^5$ cells/well into a 24-well plate and incubated over night. The next day, J16 cells were treated with 600 mJ/cm² UV and cultured for additional 3 hours. The apoptotic cells were then incubated for 15 min at 37° with 10µg/ml DAC5 F(ab')₂ fragments or left untreated, and overlaid onto the DCs at a ratio of 1:1. After 6 hours, 0,1 µg/ml LPS (Sigma) was added to a part of the cocultures. As a controls, 10 µg/ml DAC5 F(ab')₂ fragments alone and necrotic cells that had been heated 10 min to 58°C were cocultered with DCs as well. After 24h and 48h the cells were harvested, centrifuged and stained with antibodies against HLA-DR, CD86, CD83, CD11c (all from Caltag) and CCR7 (R&D) in PBS/10% mouse serum to block Fc-Receptor interactions. Cells were analyzed on a FACScan cytometer (Becton Dickinson). The supernatants were collected and stored at - 20 °C for further cytokine analysis with ELISAs for TNFα, IL-12 and IL-10 (all from BD).

Description of the Figures

FIGURE 1. DAC5 binds to the surface of apoptotic cells

a) Jurkat T cells were induced to undergo apoptotis by addition of 1 µM staurosporine (sts). At different time points, $2x10^5$ cells per sample were incubated with 100µl DAC5 supernatant or 1 µg/ml IgG2a isotype control Ab (iso), followed by incubation with FITC-labelled anti-mouse IgG antibodies. To discriminate between early and late apoptosis, aliquots were incubated in parralel with FITC-labelled Annexin V (AxV) or 2 µg/ml propidium iodide (PI). The kinetic is representative for 3 experiments. Inset shows a representative DAC5 staining after 4 hours incubation with staurosporine (shaded histogram, in comparison to DAC5 staining on live cells.

b) Jurkat T cells were irradiated with 150 Gy and cultured for additional 6 hours. Alternatively, cells were incubated for 6 hours with 1 µM staurosporine (sts) or 1 µg/ml leucine zipper CD95 ligand (LZ-CD95L). Subsequently, cells were stained with FITC-labeled Annexin V or FITC-labeled DAC5 in Annexin Binding buffer.

FIGURE 2. DAC5 precipitates Annexin I

a) Postnuclear lysates of 10^7 CEM cells were immmunoprecipitated with Protein A sepharose and purified DAC5 or IgG2a isotype control (iso), or protein A sepharose alone (-). Precipitates were resolved on a 12% SDS PAGE and silver stained. The protein bands labeled

"Annexin I" were cut from the gel and subjected to tryptic digest, followed by analysis via mass spectroscopy. Results obtained by comparison with the xxx database and peptides were identified as derived from Annexin I. hc − heavy chain; lc − light chain.

5

FIGURE 3. Annexin I is bound to the outside of apoptotic cells

$5x10^6$ CEM cells were treated with 1 µM sts for different time points and centrifuged to obtain the supernatant. The pellets were washed with PBS/EDTA to yield the membrane bound Annexin I (membrane). Finally,

10   cell pellets were lysed and aliquods of all fractions were resolved on a 12% SDS PAGE followed by detection with monoclonal anti-Annexin I Abs. Westernblot shown is representative for 3 experiments.

FIGURE 4. Annexin I facilitates apoptotic cell engulfment and

15   ### suppresses DC cytokine secretion

$2x10^5$ d7 immature DCs were incubated with $2x10^5$ DiD-labelled apoptotic Jurkat cells and cocultured for 24h or 48h. To part of the cultures 0,1 µg/ml LPS was added after 6h. Cells were then harvested, stained with antibodies against CD11c, CD83, CD86 and HLA-DR and analyzed by

20   flowcytometry (a,c). The supernatants were collected and analyzed with ELISAs for TNFα and IL-12 (b).

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Claims

1. Anti-Annexin antibody.

2. Anti-Annexin antibody, which is an anti-Annexin I antibody.

3. Antibody according to claims 1 or 2, wherein the antibody is labeled.

4. Antibody according to the preceding claims, wherein the label is a molecule that allows detection of the antibody in vivo or in vitro.

5. Antibody according to one of the preceding claims, wherein the label is an effector molecule, especially a toxic substance or a radioactive substance.

6. Use of an antibody according to at least one of claims 1 to 5 for the detection of apoptosis in vivo.

7. Use of an antibody according to at least one of claims 1 to 5 for the diagnosis of diseases linked to an increased apoptosis, especially diabetes and auto-immune diseases or cardiovascular and vascular diseases.

8. Use of an antibody according to at least one of claims 1 to 5 for blocking anti-inflammatory signals on cells.

9. Use of an antibody according to at least one of claims 1 to 5 for cancer therapy or for supporting a common cancer therapy.

10. Use of an annexin to avoid inflammatory response to tissue, especially transplantation tissue in a patient or in vitro.

11. Use according to claim 10, wherein Annexin I is used.

Abstract

In order to define new molecular structures on the surface of apoptotic but

5   not live cells, we immunized C57BL/6-mice with apoptotic Jurkat cells. Splenic B-cells of these mice were then fused with myeloma-cells to generate hybridomas, whose antibodies were screened by FACS-analysis for their ability to discriminate between live and apoptotic Jurkat cells. One of the monoclonal antibodies immunoprecipitated a protein of about 38 kD, which

10   proved to be Annexin I by protein sequencing. Further analysis confirmed that upon induction of apoptosis, Annexin I is translocated from the inside of the cell to the outer leaflet of the cell membrane, where it binds in a calcium dependent manner. The kinetics of Annexin I translocation reflect exposure of phosphatidylserine on the surface of apoptotic cells. Currently, we conduct

15   functional studies to elucidate the role of externalized Annexin I in phagocytosis and inflammation.

20

ll 24.09.2003

Fig. 1

a

Fig. 1·

b

Fig. 2

a

Fig. 3

anti Annexin I

Fig. 4

a

Fig. 4

b

TNF

IL-12

Fig. 4
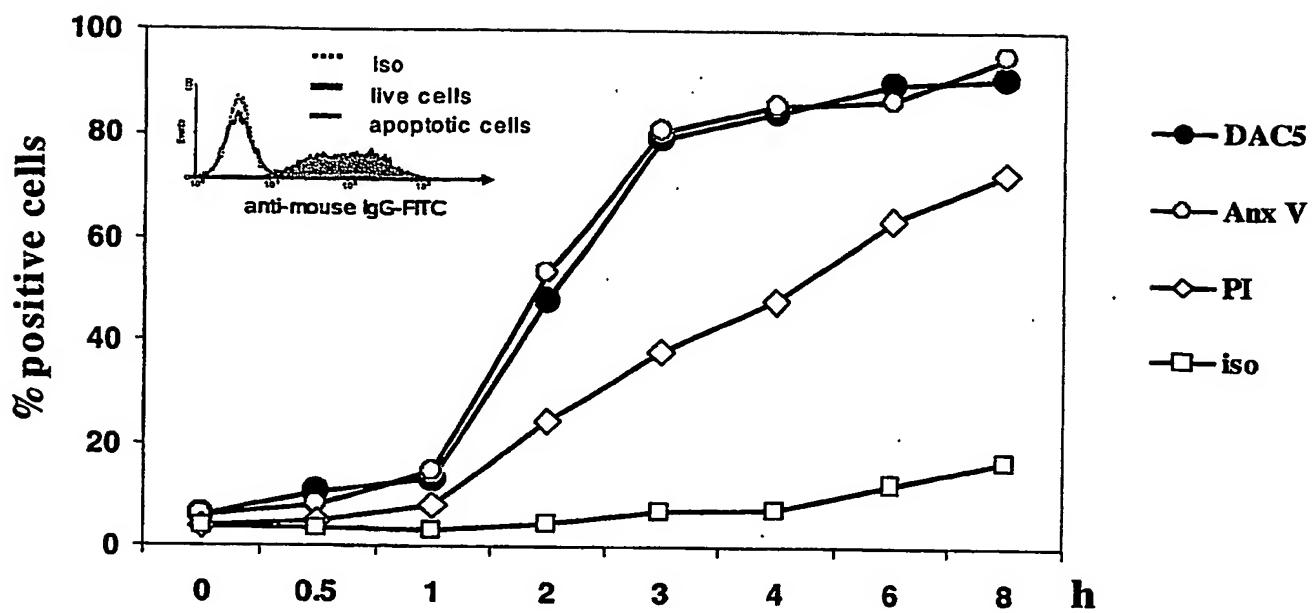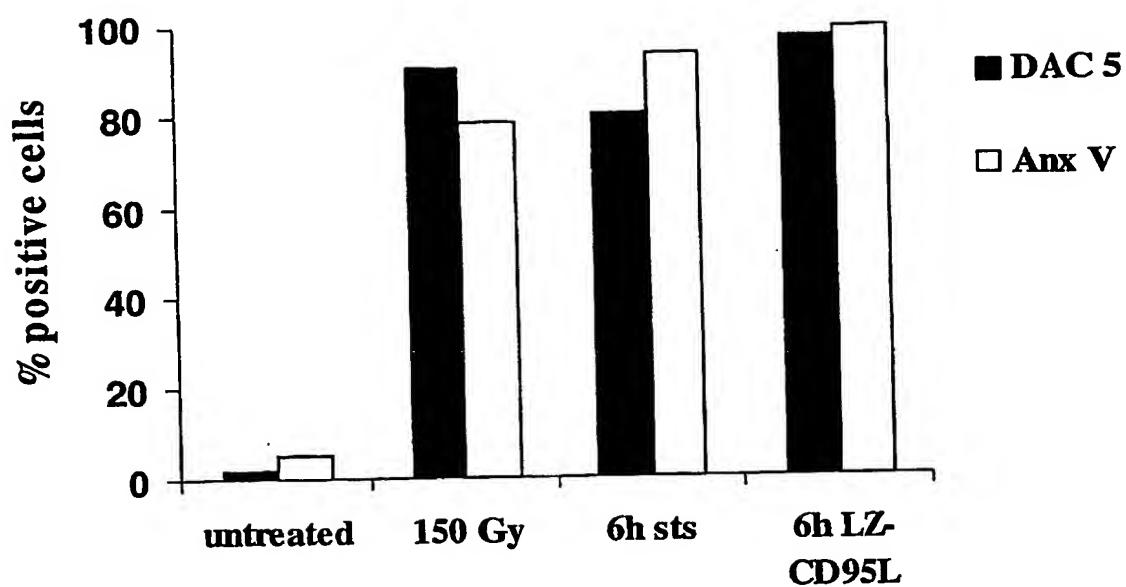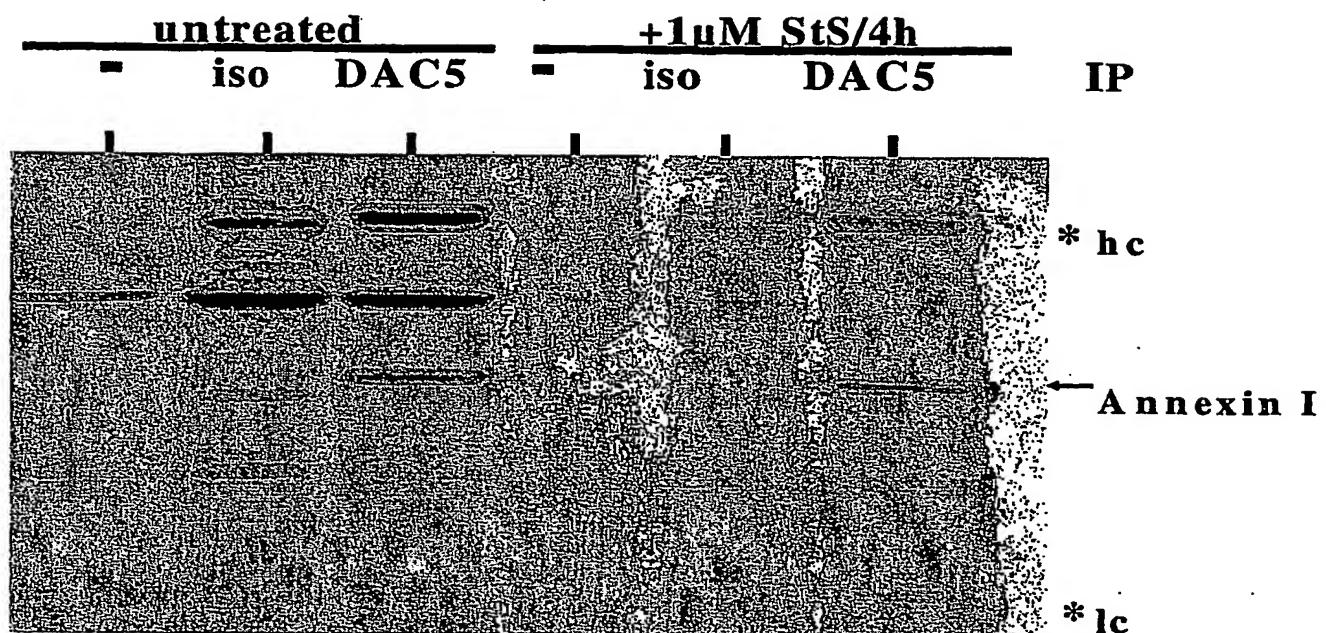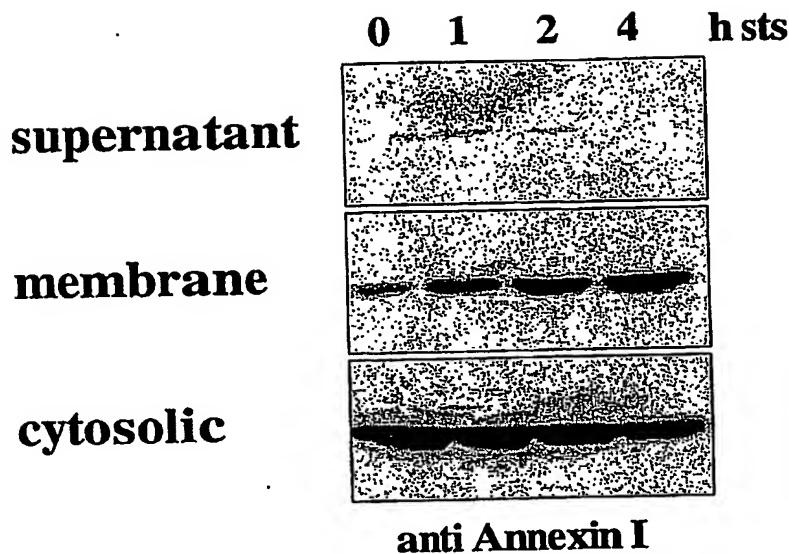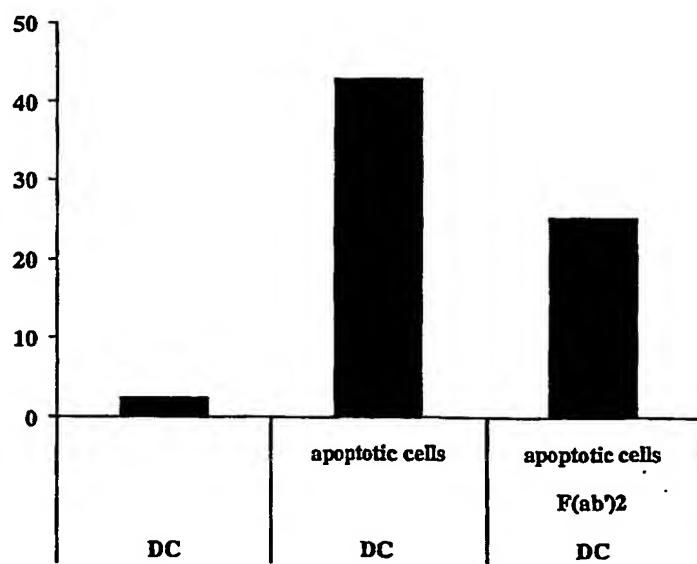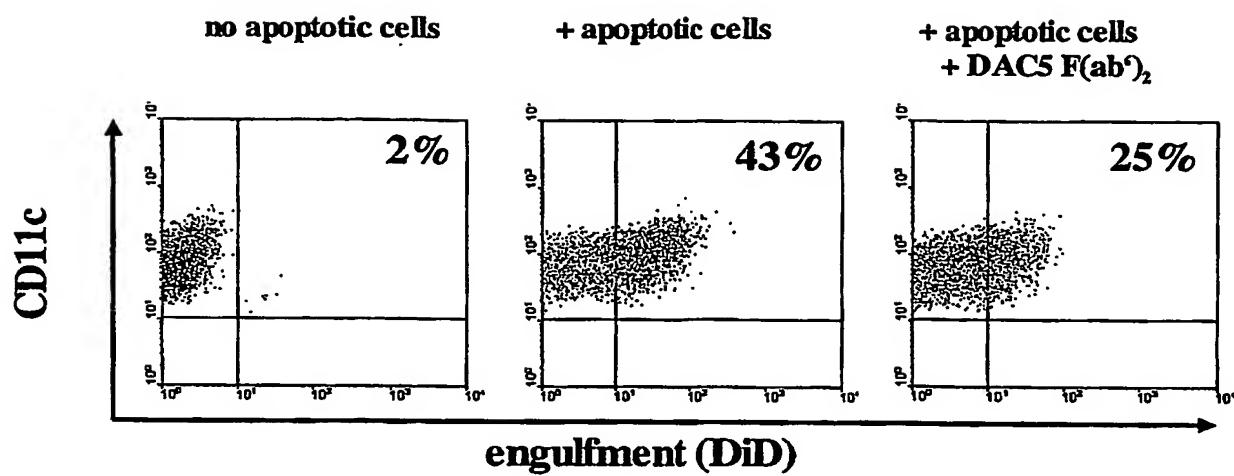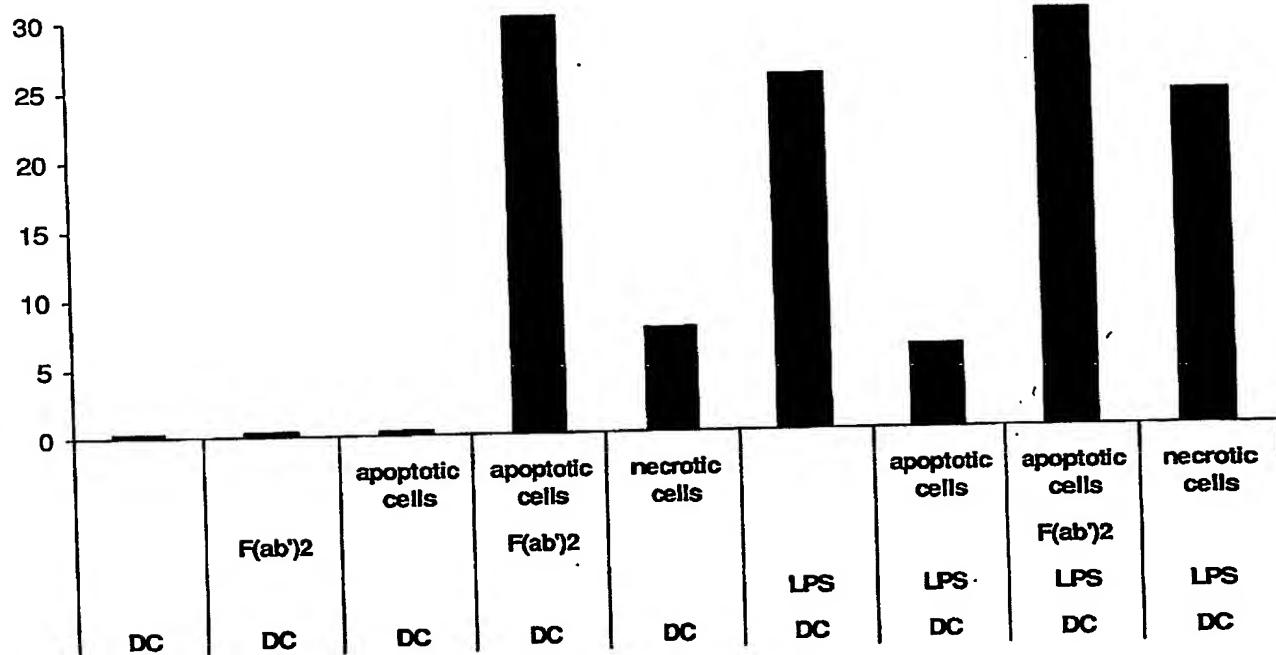
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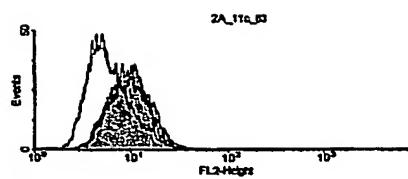
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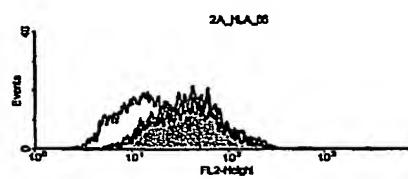
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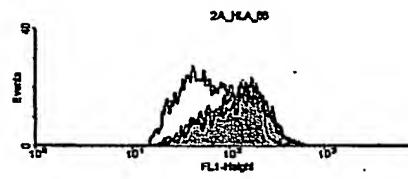
— DC without engulfed cells
— DC with engulfed apoptotic cells
— DC with engulfed apoptotic cells + DAC 5 F(ab')₂
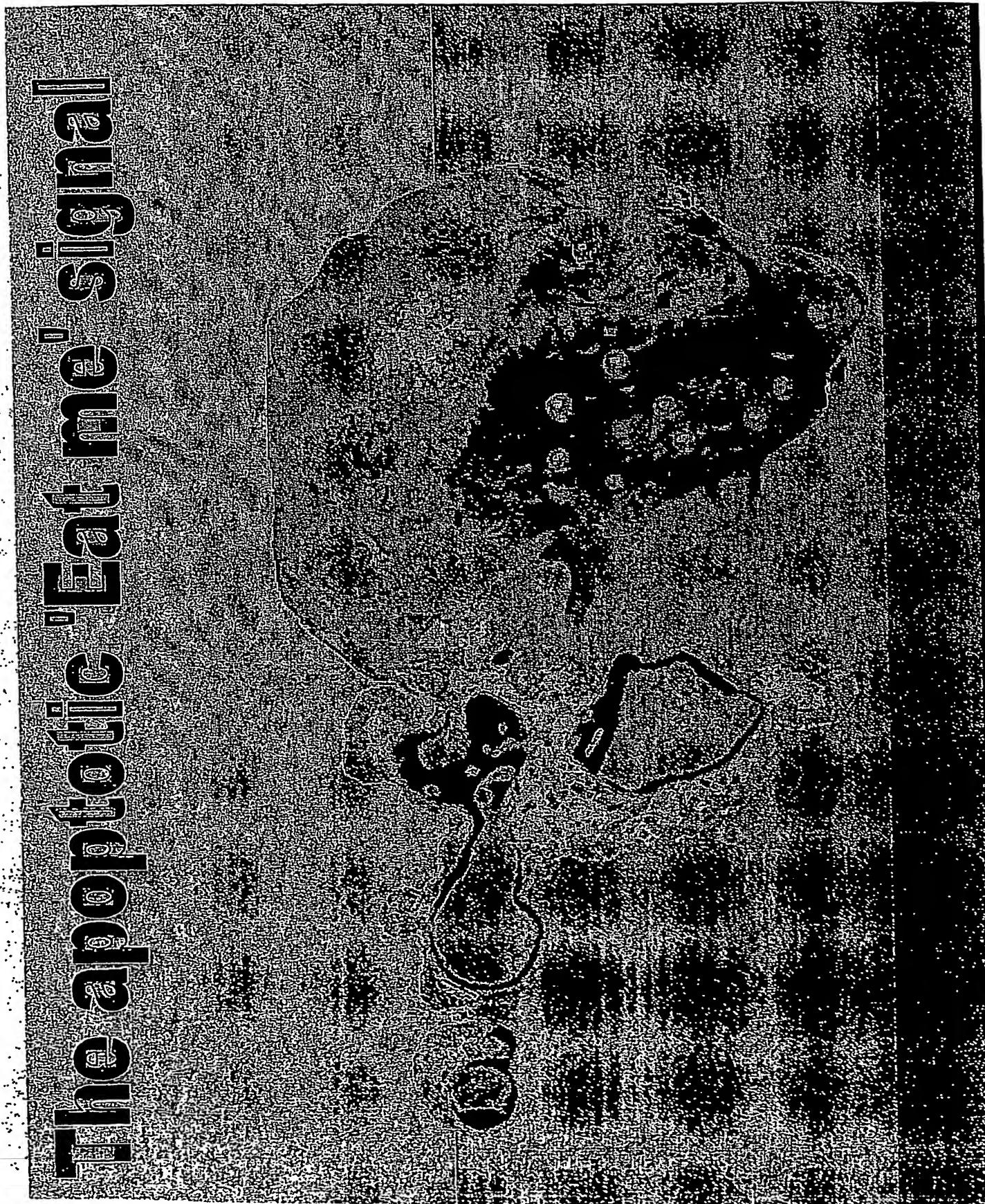
The apoptotic 'Eat me' signal

Potential 'Eat Me' Signals

PtdSer

fig 1

Potential ‚Eat Me‘ Signals

PtdSer

Annexin V

A Detector of Apoptotic Cells (DAC)

Immunization w/ apoptotic Jurkat-cells
Stimulus: 1μM Staurosporin

Screen supernatants
• ~600 supernatants/spleen
• ~9500 supernatants screened
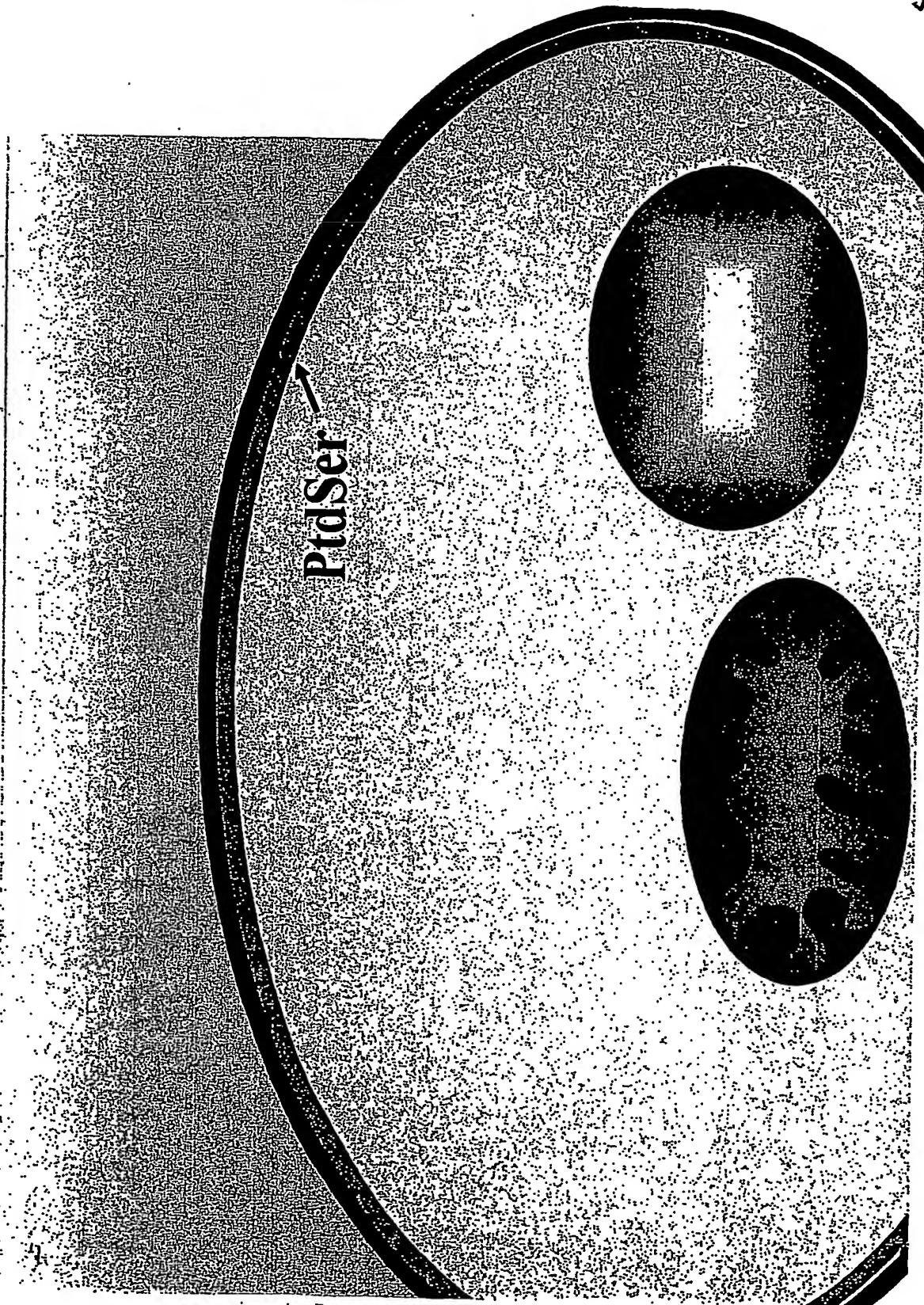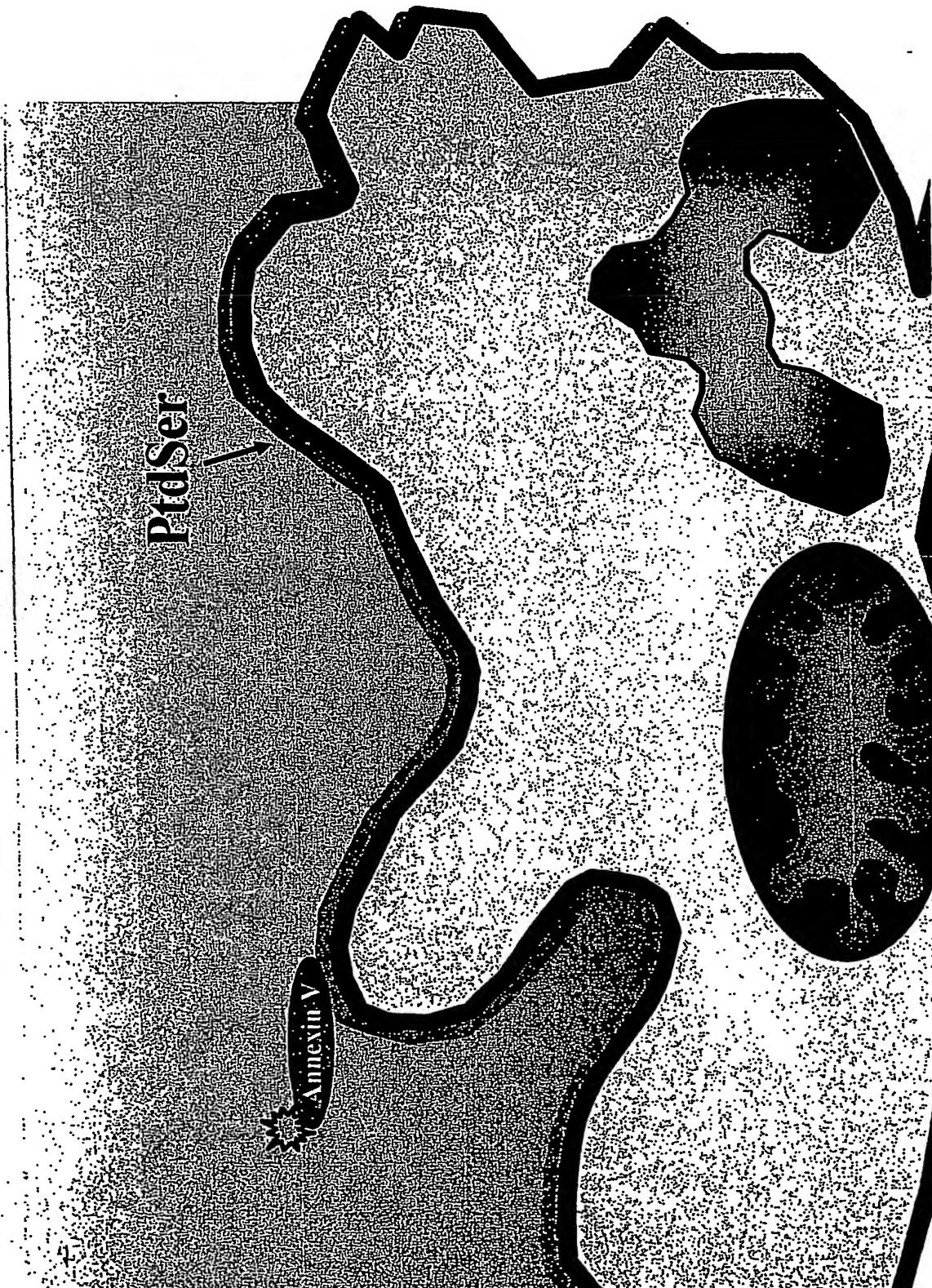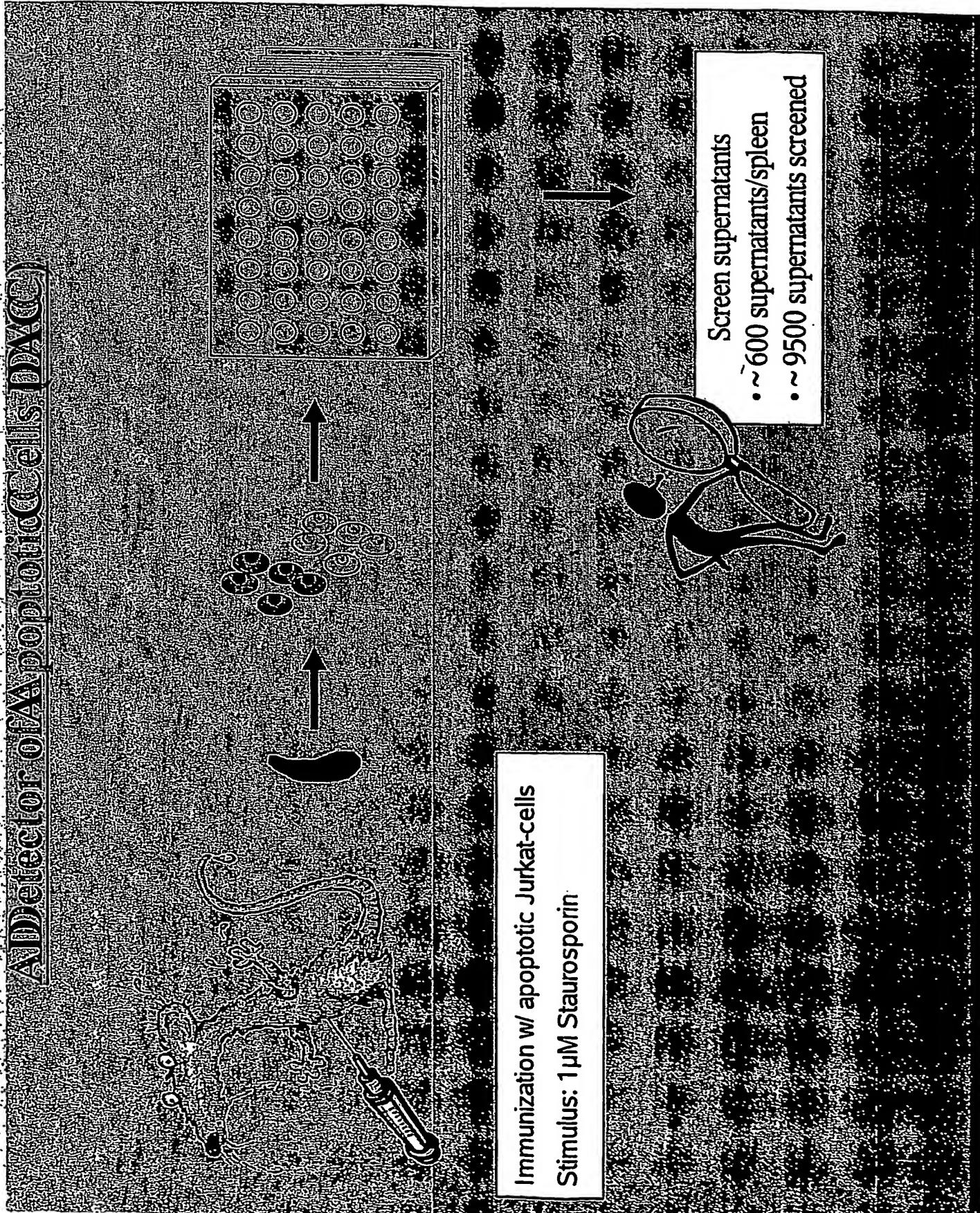
Binding of DAC5 to apoptotic cells

cell type	DAC5	PtdSer
J16 +Sts	+	+
CEM +Sts	+	+
T cells +Sts	+	+
HeLa +Sts	+	+

anti-IgG-FITC

- unstained
- isotype IgG2a
- live CEM
- apoptotic CEM

Fig 10

DAC 5 binds early to apoptotic cells

Fig 11

DAC5-IP

silver stain

untreated   +1µM StS/4h

Iso DAC5   Iso DAC5

Marker

Iso DAC5 - IP

heavy chain

Annexin I

light chain

12 % SDS

Fig 12
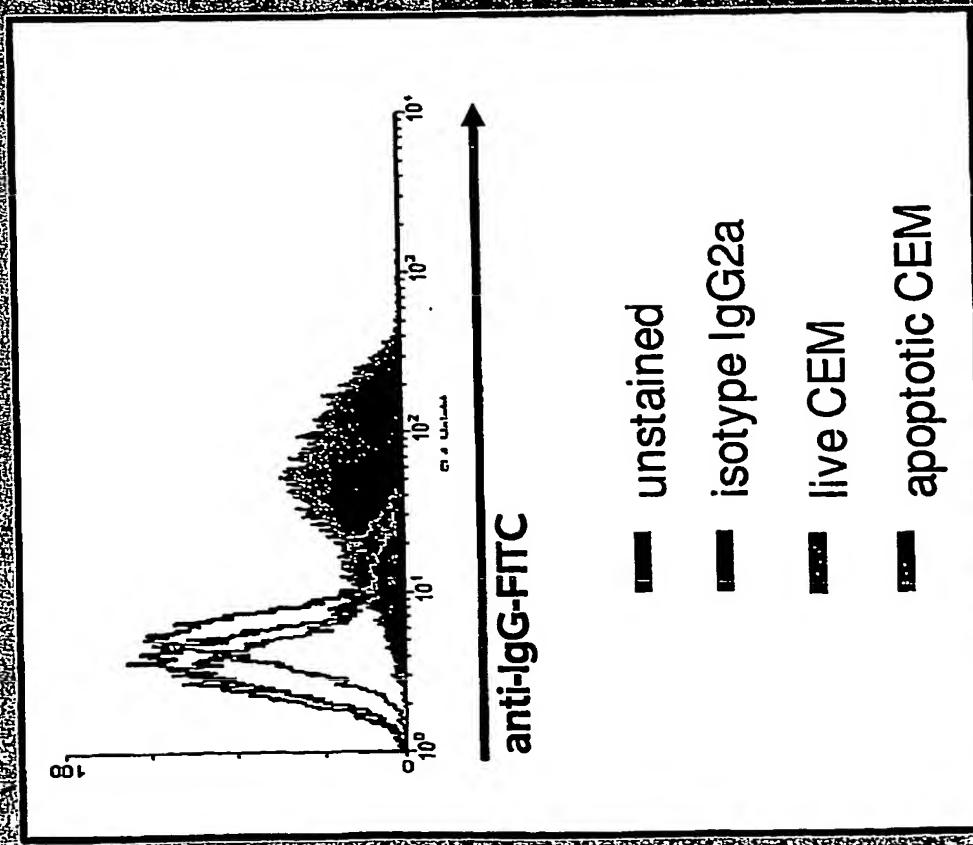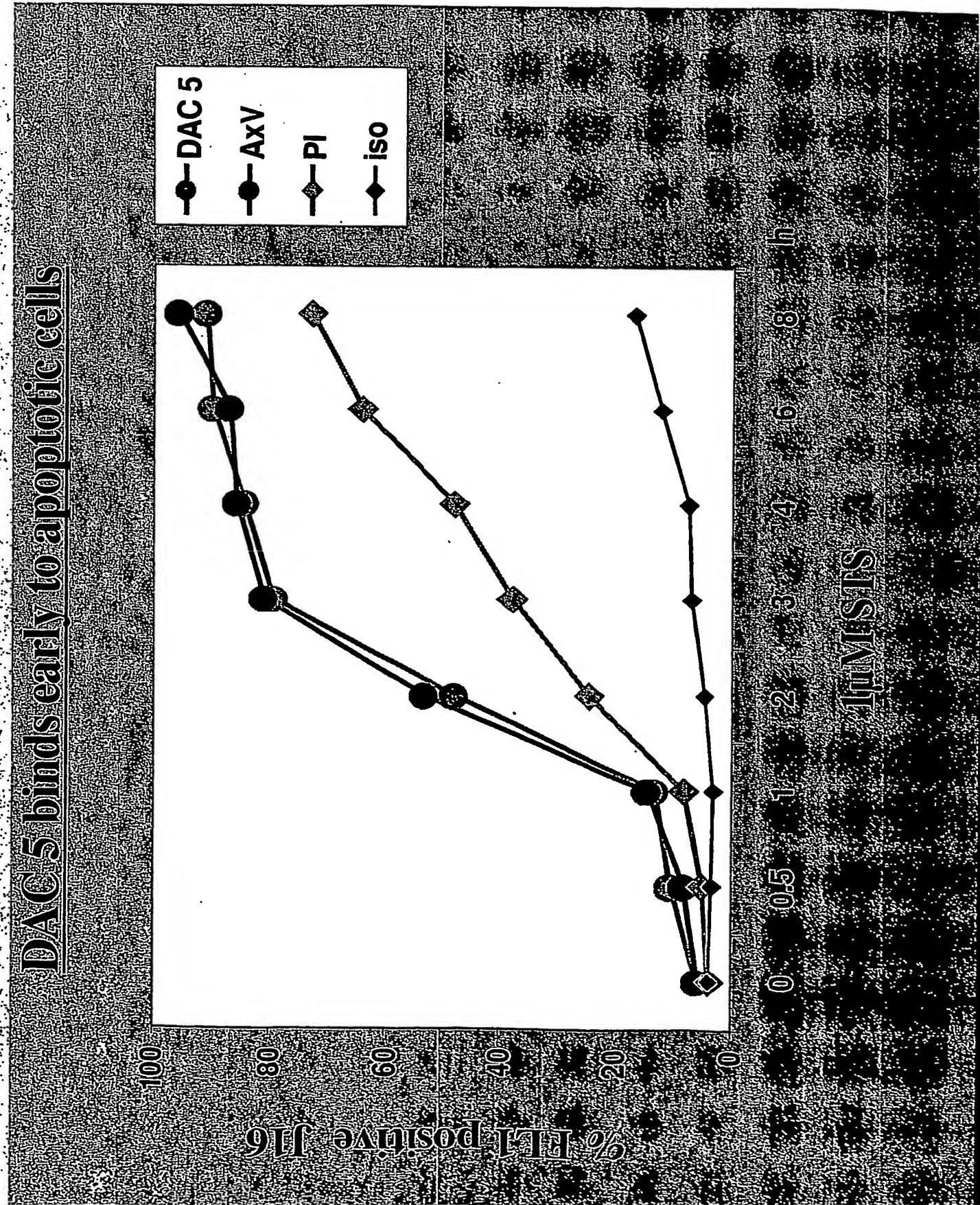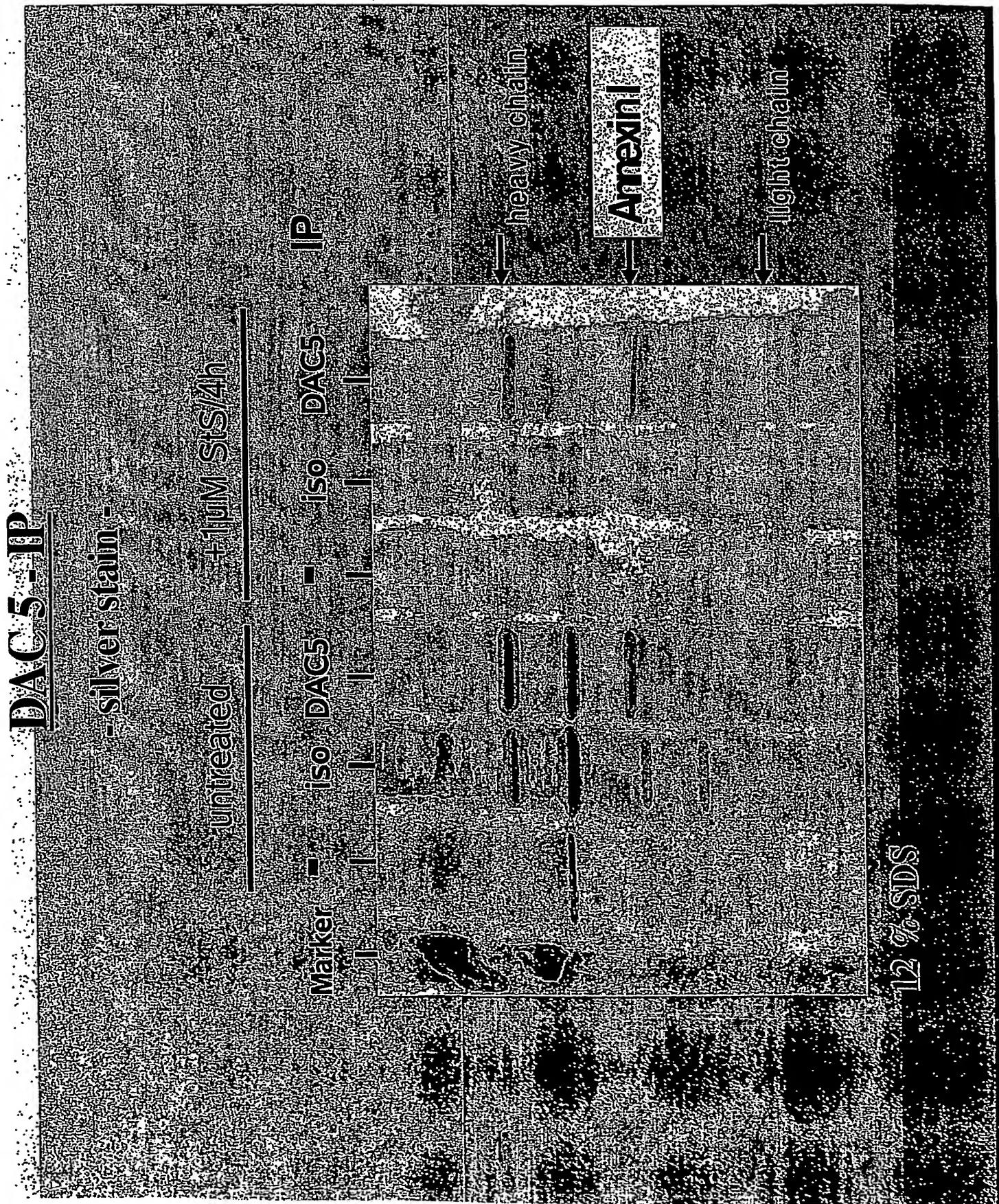
Annexins on the Cell Membrane
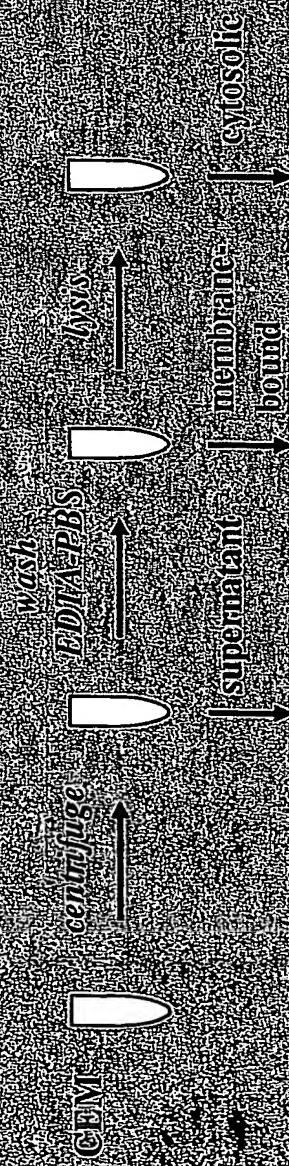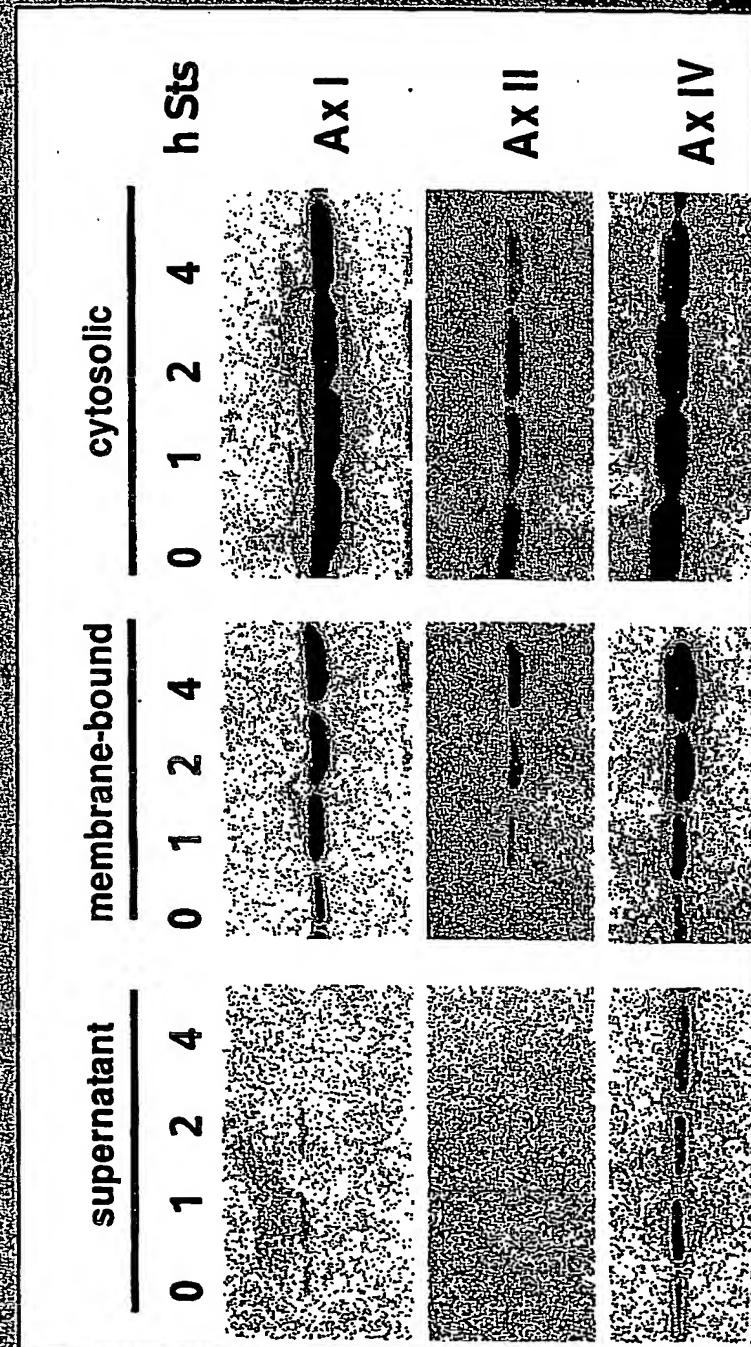
Fig 13
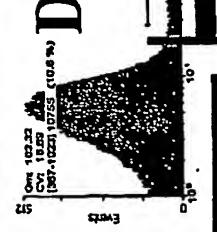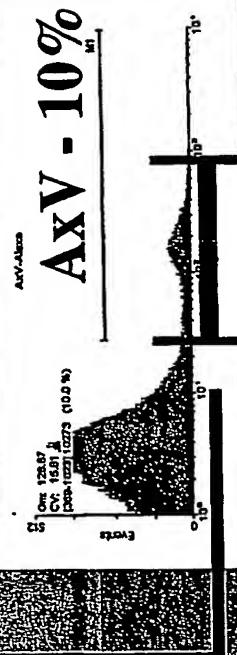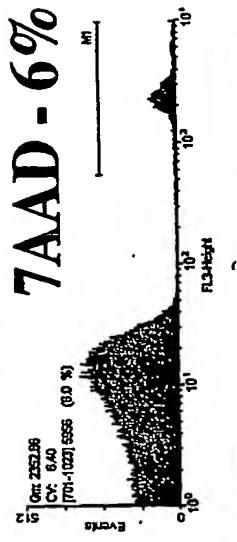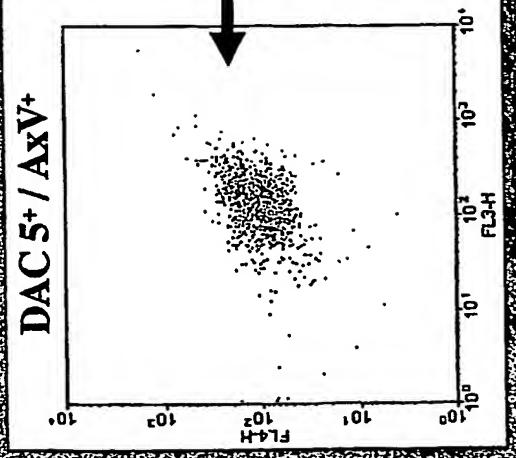
Ax1on Thymocytes
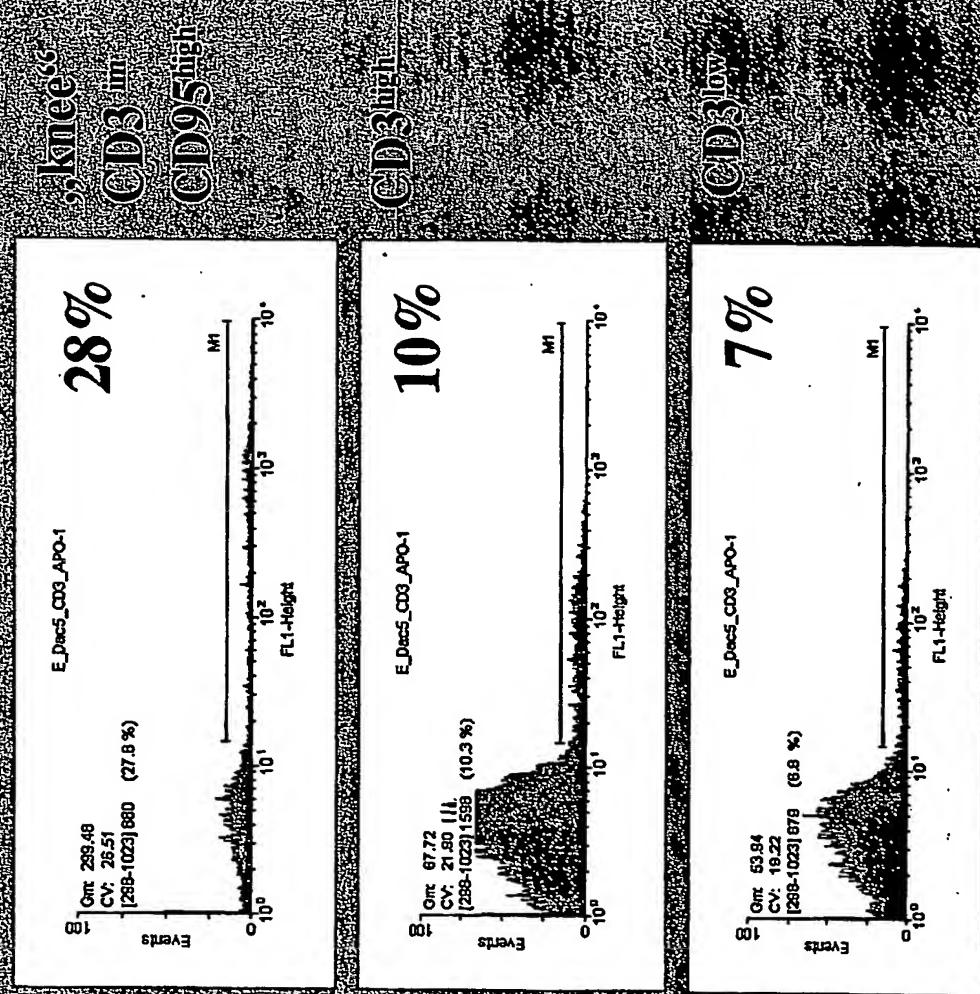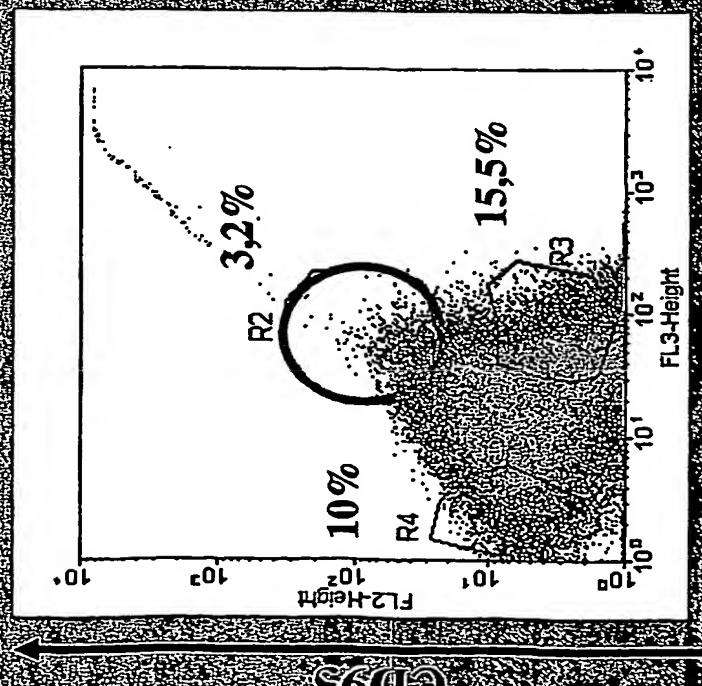
17126

Fig 14

18126

Fig 15

Preparation of Dendritic Cells

Fig 16

Annexin I is an "eat me" signal

Apoptotic Cells do not induce DC Cytokine Secretion

TNFα
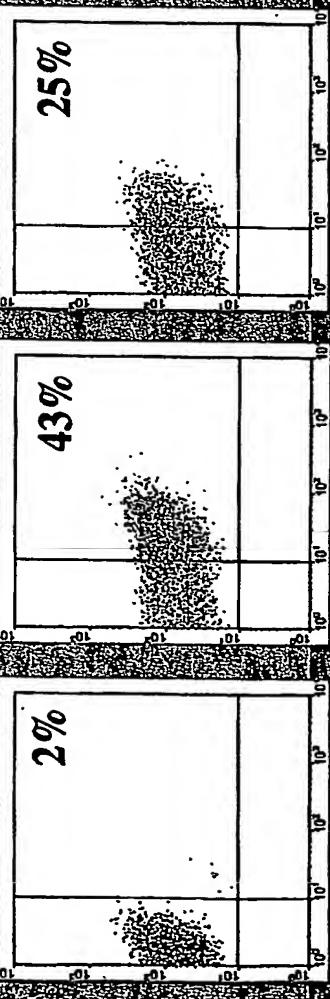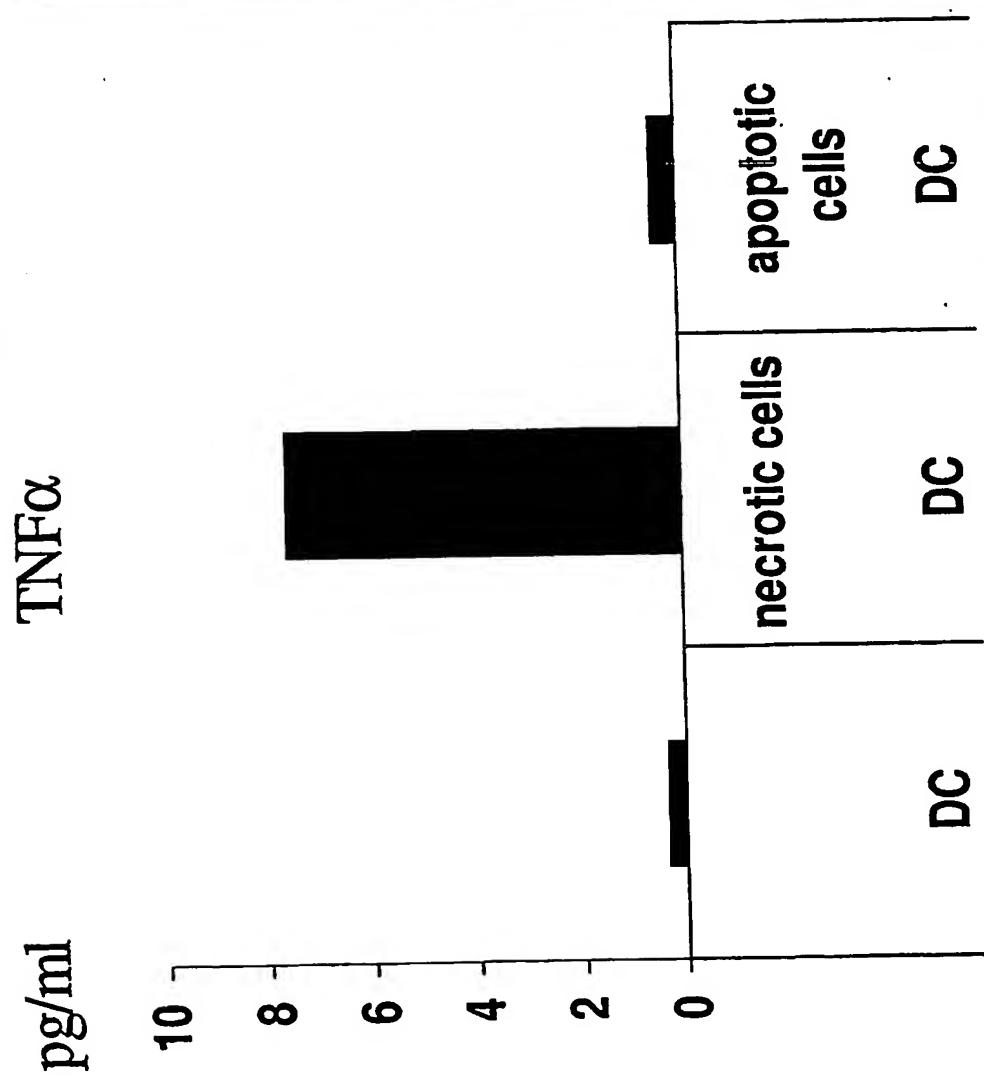
Fig 18

Apoptotic Cells inhibit DC Cytokine Secretion
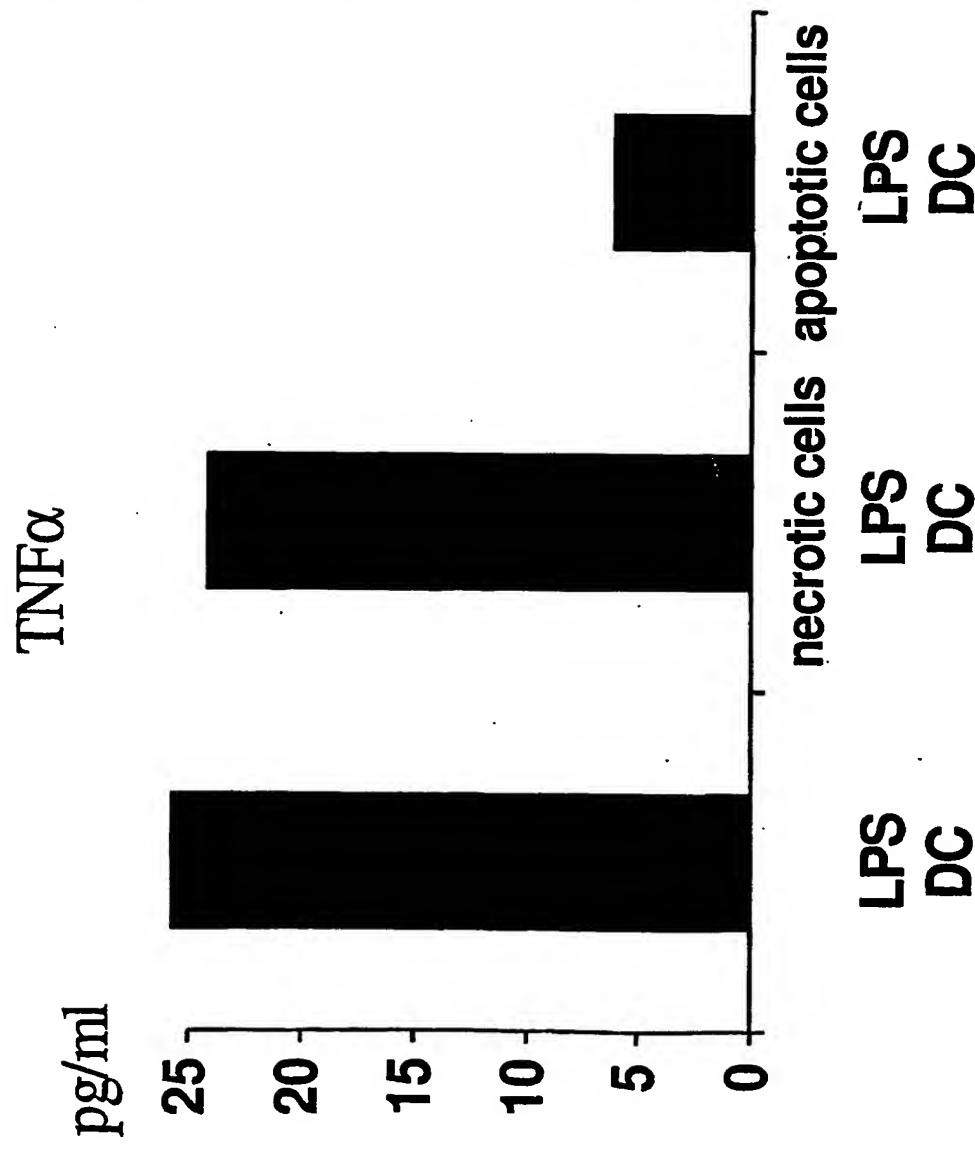
Fig 19

Annexin II is a tolerogenic signal

TNFα

Fig 20
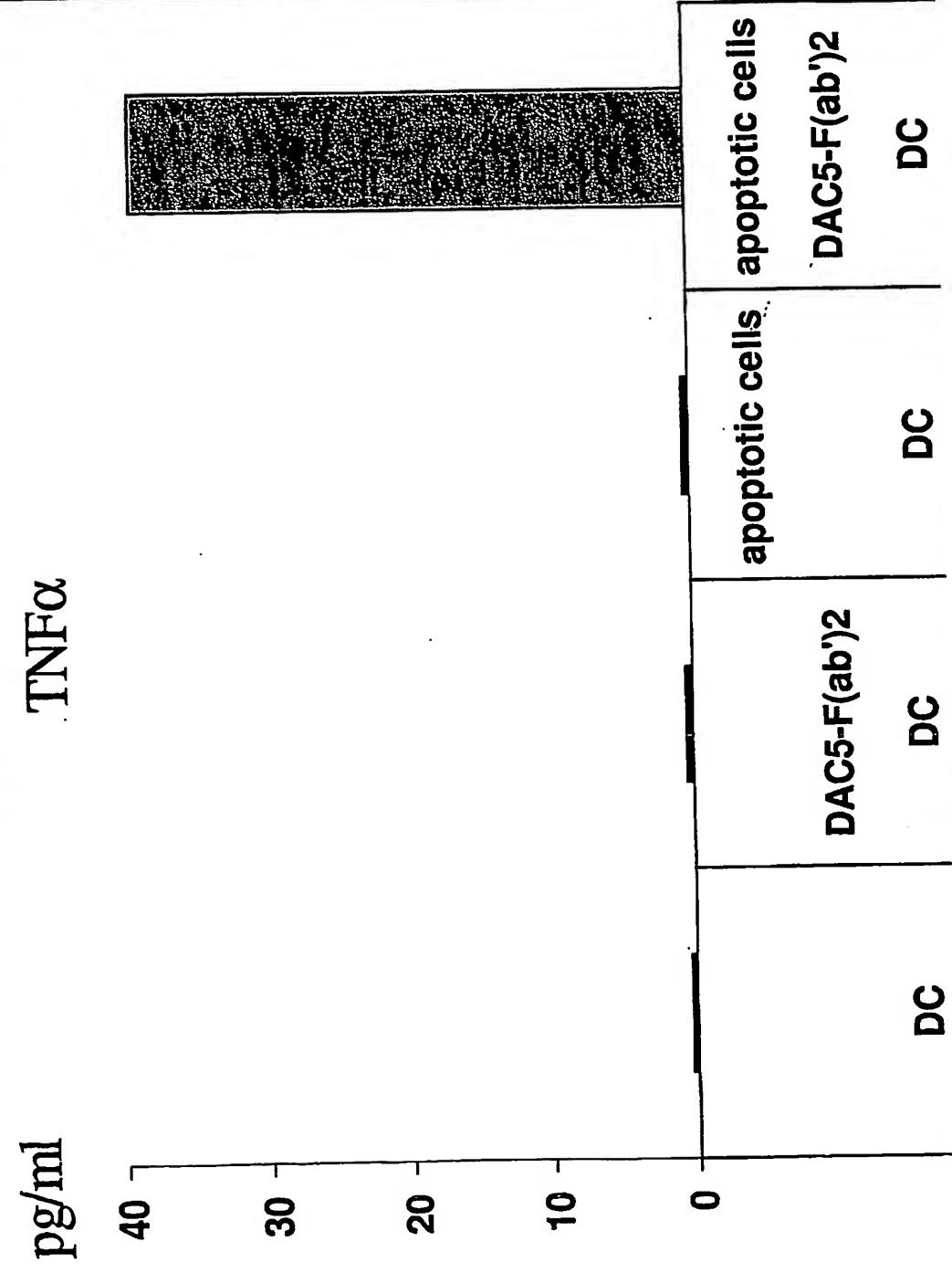
Conclusion

Our data suggest that apoptotic cells maintain a through a tolerogenic signal mediated by suppressing
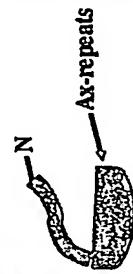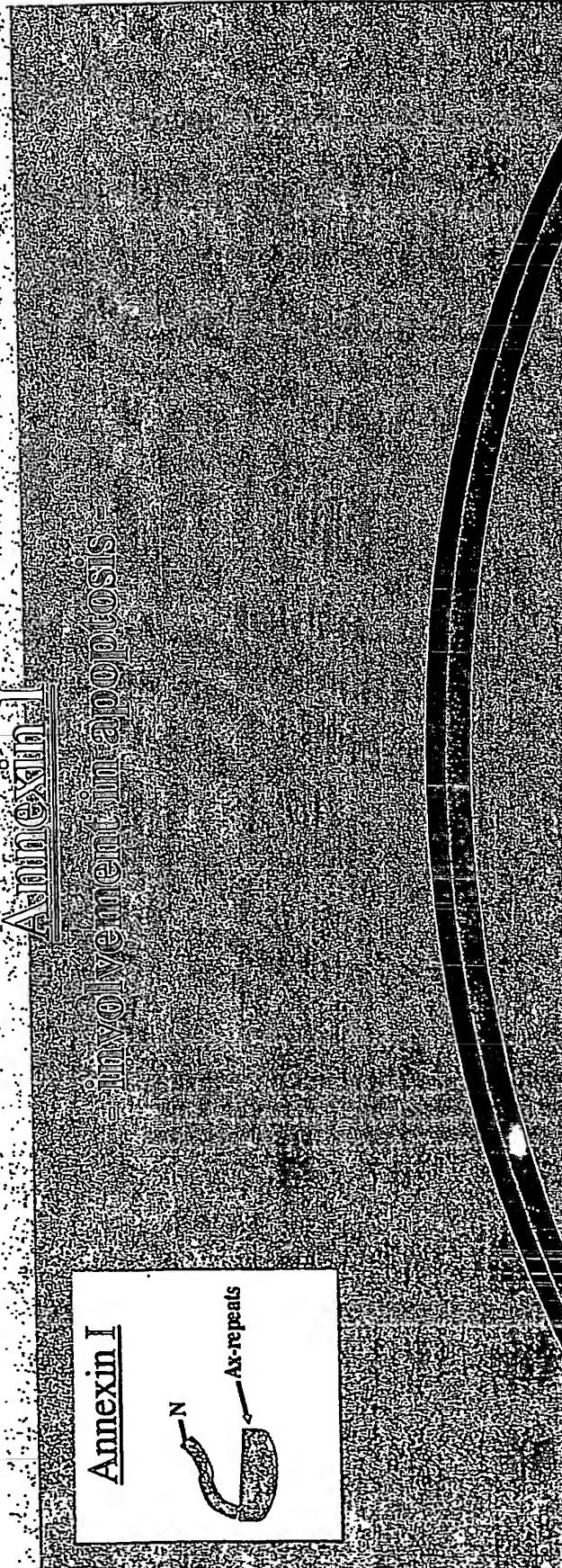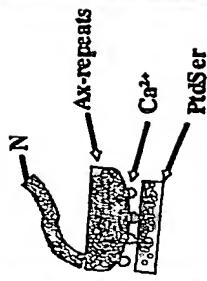
fig 6

Annexin I

Involvement in apoptosis

Annexin I

N

Ax-repeats

Ca2+

Fig 2.

Annexin I — involvement in apoptosis —

Annexin I

N — Ax-repeats — Ca²⁺ — PtdSer
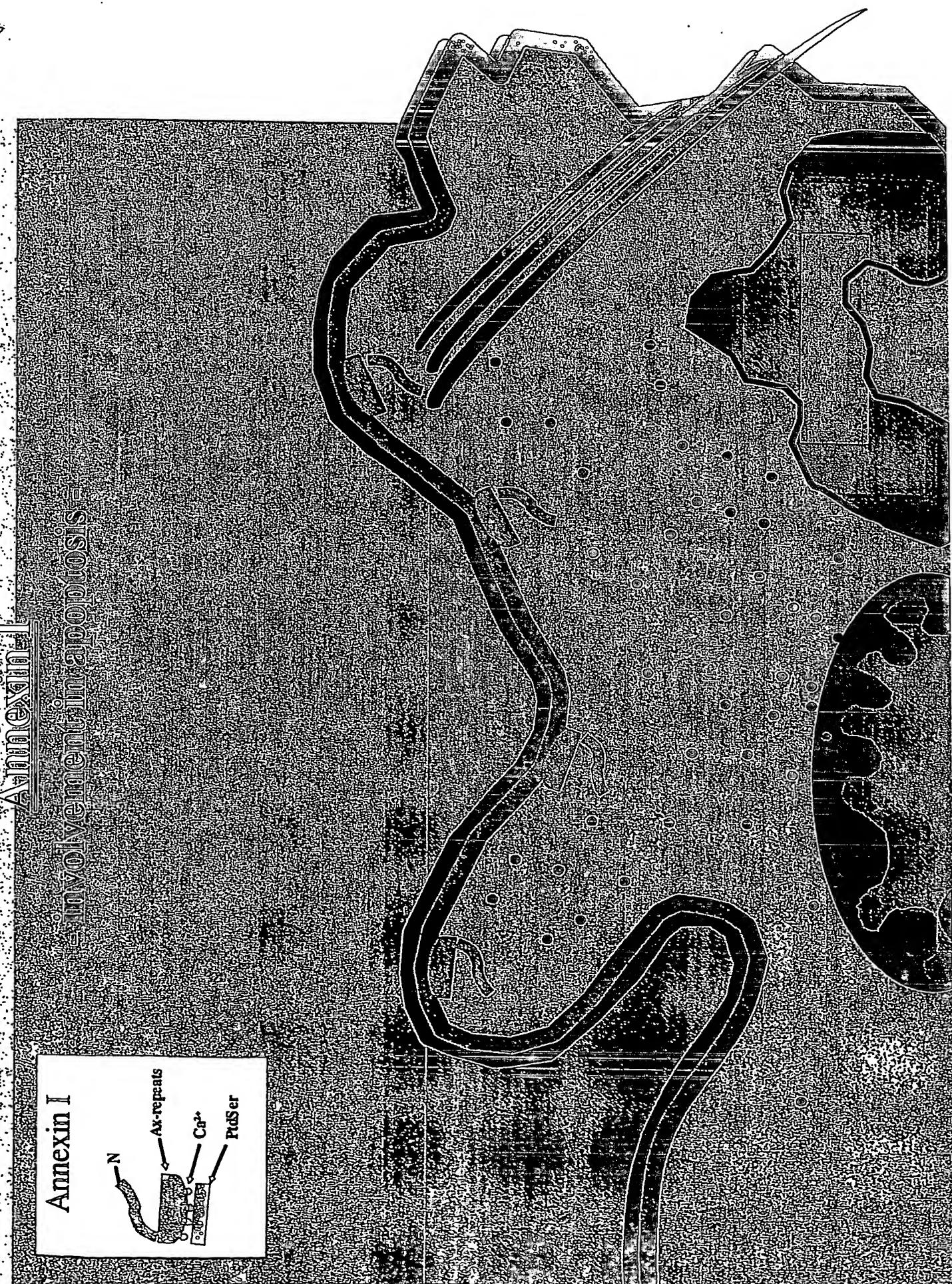
Fig 23

Annexin I

– involvement in apoptosis –

Involvement of
Other Annexins (II/IV)?

DACS

Phagocytosis
anti-inflammation